Figure 1: Cloning by *in vitro* assembly and transformation of competent Bacillus

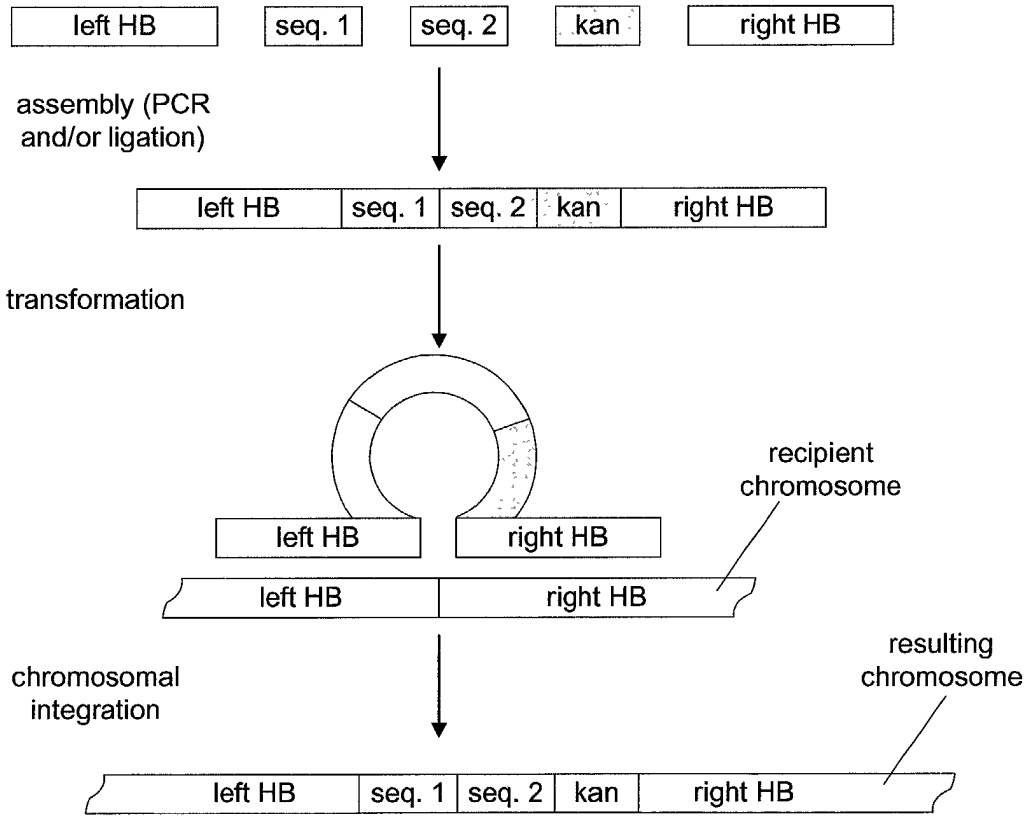


Figure 2: Adding non-homologous flanks increases the transformation efficiency.

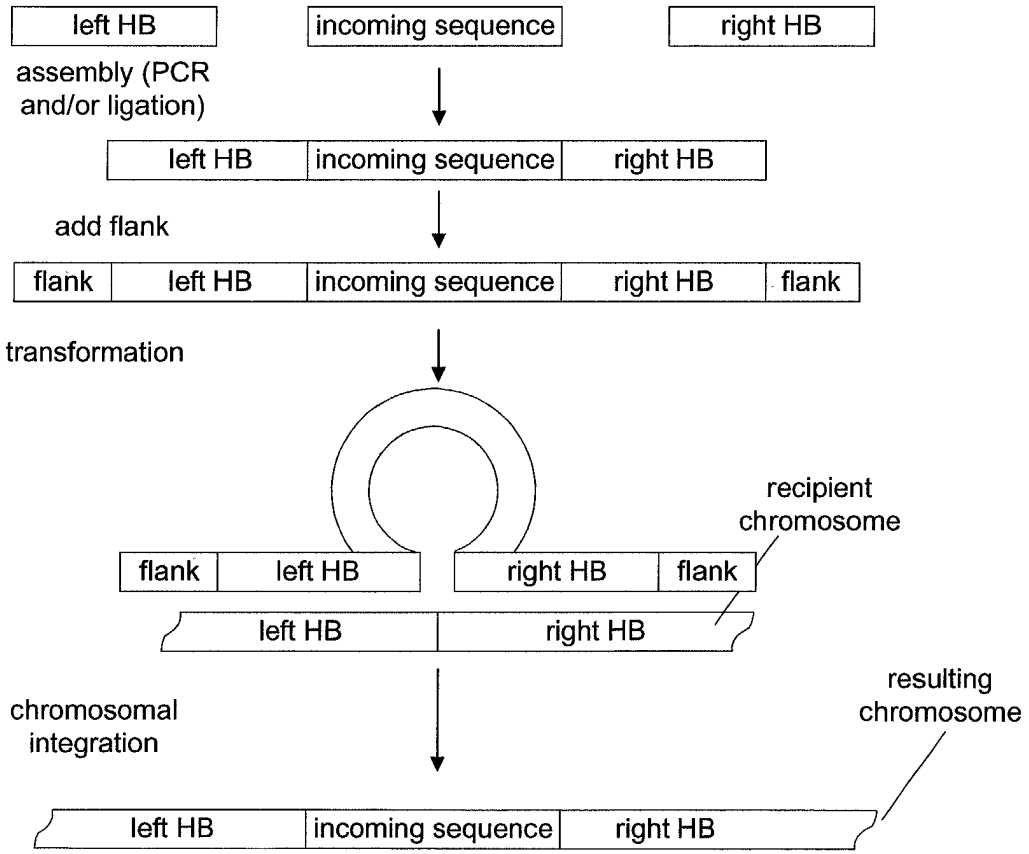


Figure 3: PCR mutagenesis of a region of the Bacillus chromosome

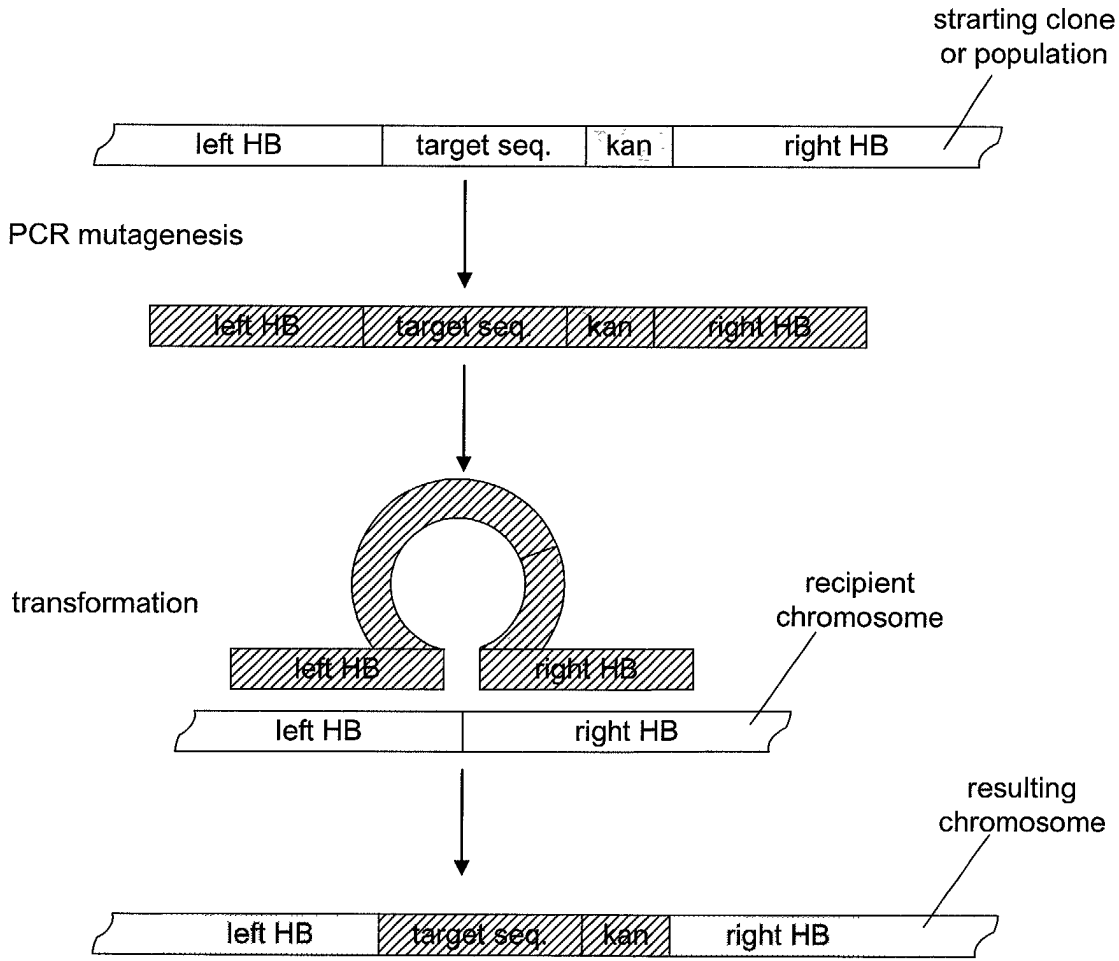


Figure 4: Maximizing the homology between the transforming DNA and the target region of the chromosome increases the transformation efficiency.

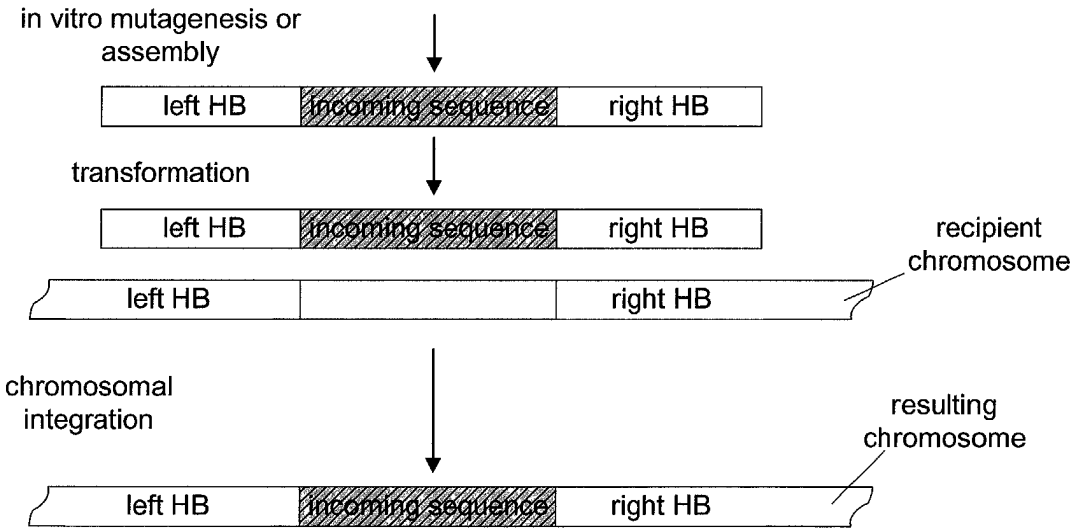


Figure 5: Using a competent host that carries an inactive version of the marker gene, used to select transformants

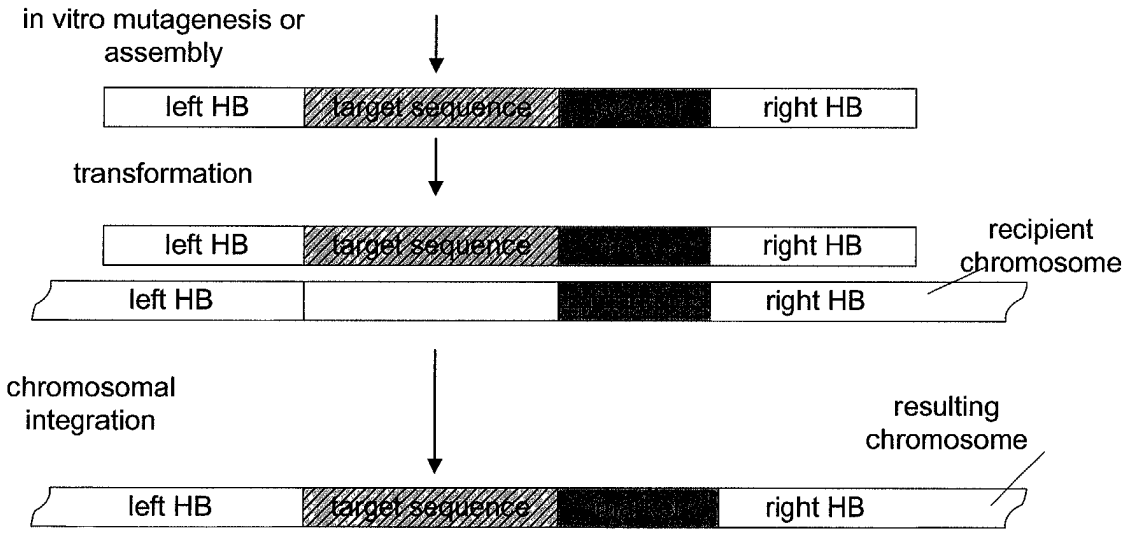


Figure 6: General structure of transforming DNA

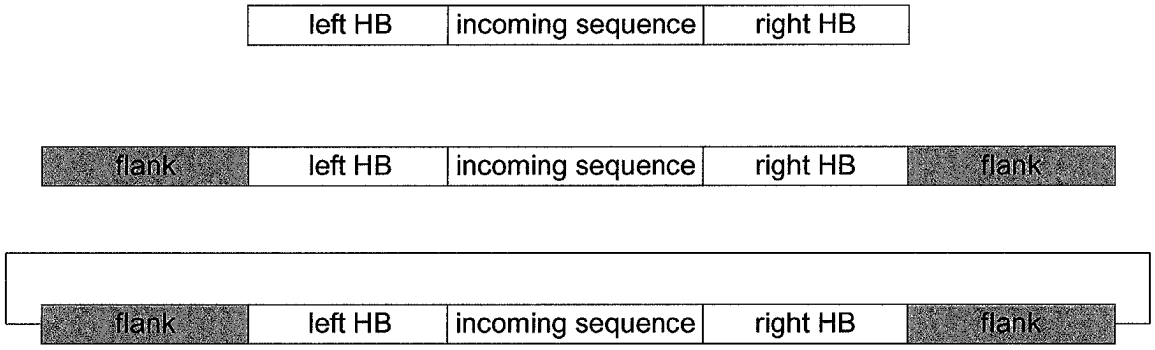
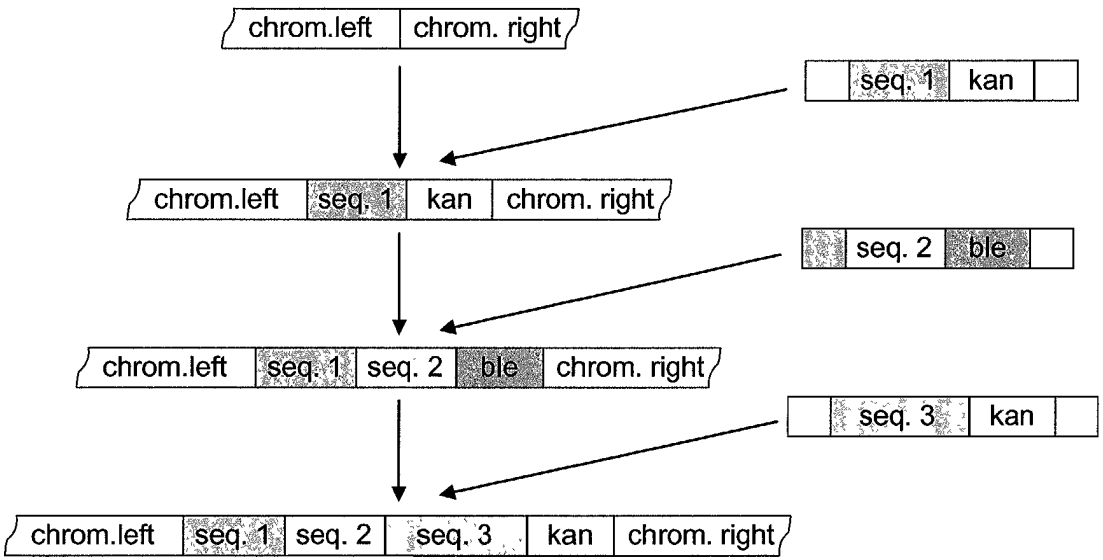


Figure 7: Bacillus strain construction by iterative marker replacement



Advantages

- Replacement allows one to verify that construct went into target locus.
- Large sequences can be assembled in vivo.
- Avoids repeat PCR amplification
- Entire construct can be moved by transduction or transformation.
- Two markers are sufficient to introduce an unlimited number of genes.

Figure 8A

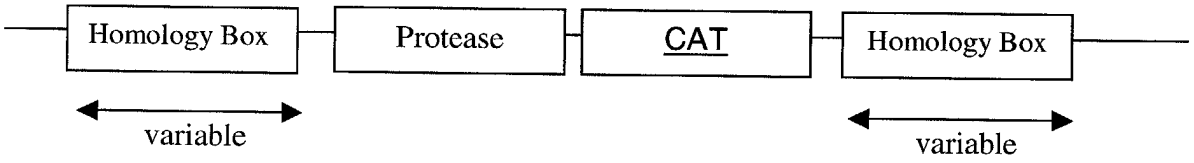
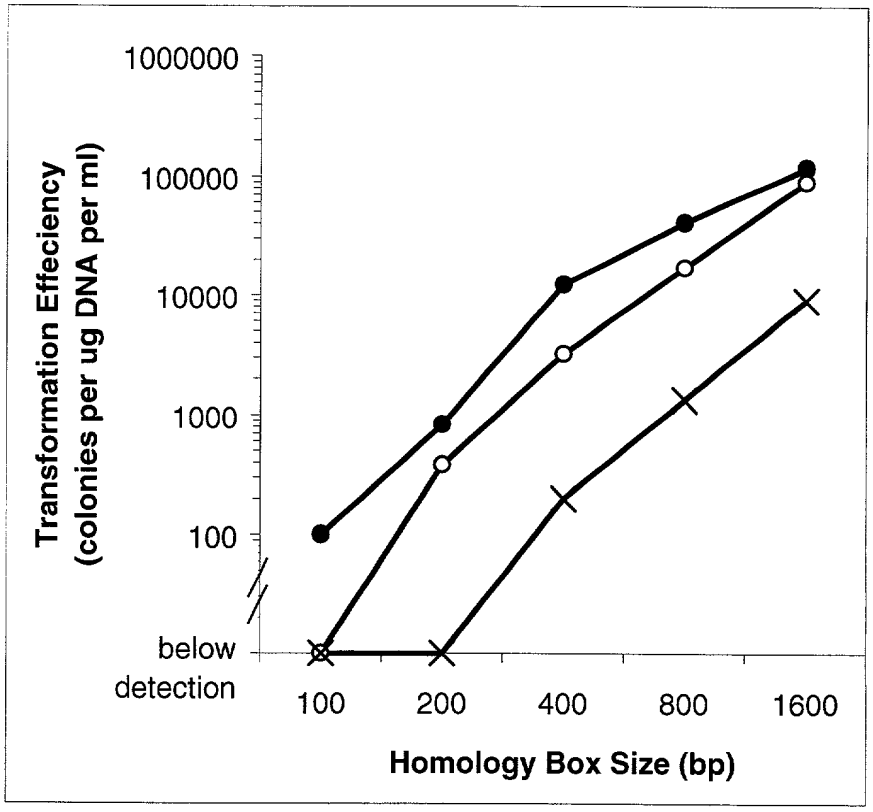


Figure 8B. Optimization of homology box size



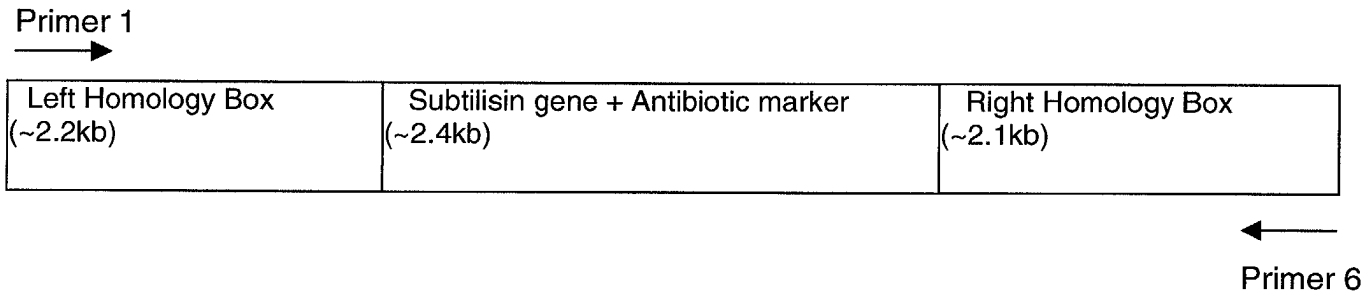

Primer 1
Left Homology Box
(~2.2kb)
Subtilisin gene + Antibiotic marker
(~2.4kb)
Right Homology Box
(~2.1kb)
Primer 6

Figure 9

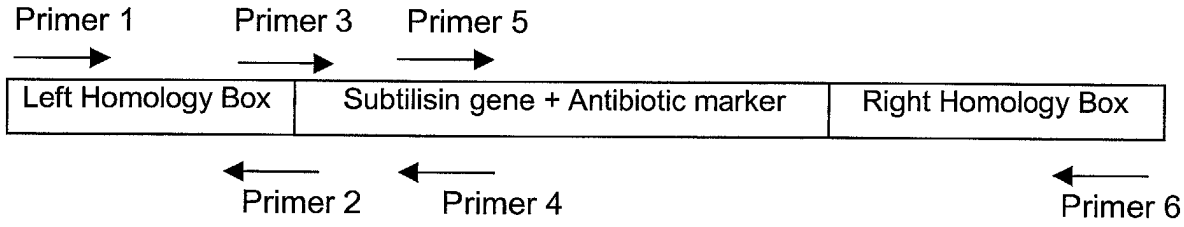

Primer 1
Primer 3
Primer 5
Left Homology Box
Subtilisin gene + Antibiotic marker
Right Homology Box
Primer 2
Primer 4
Primer 6

Figure 10

Figure 11

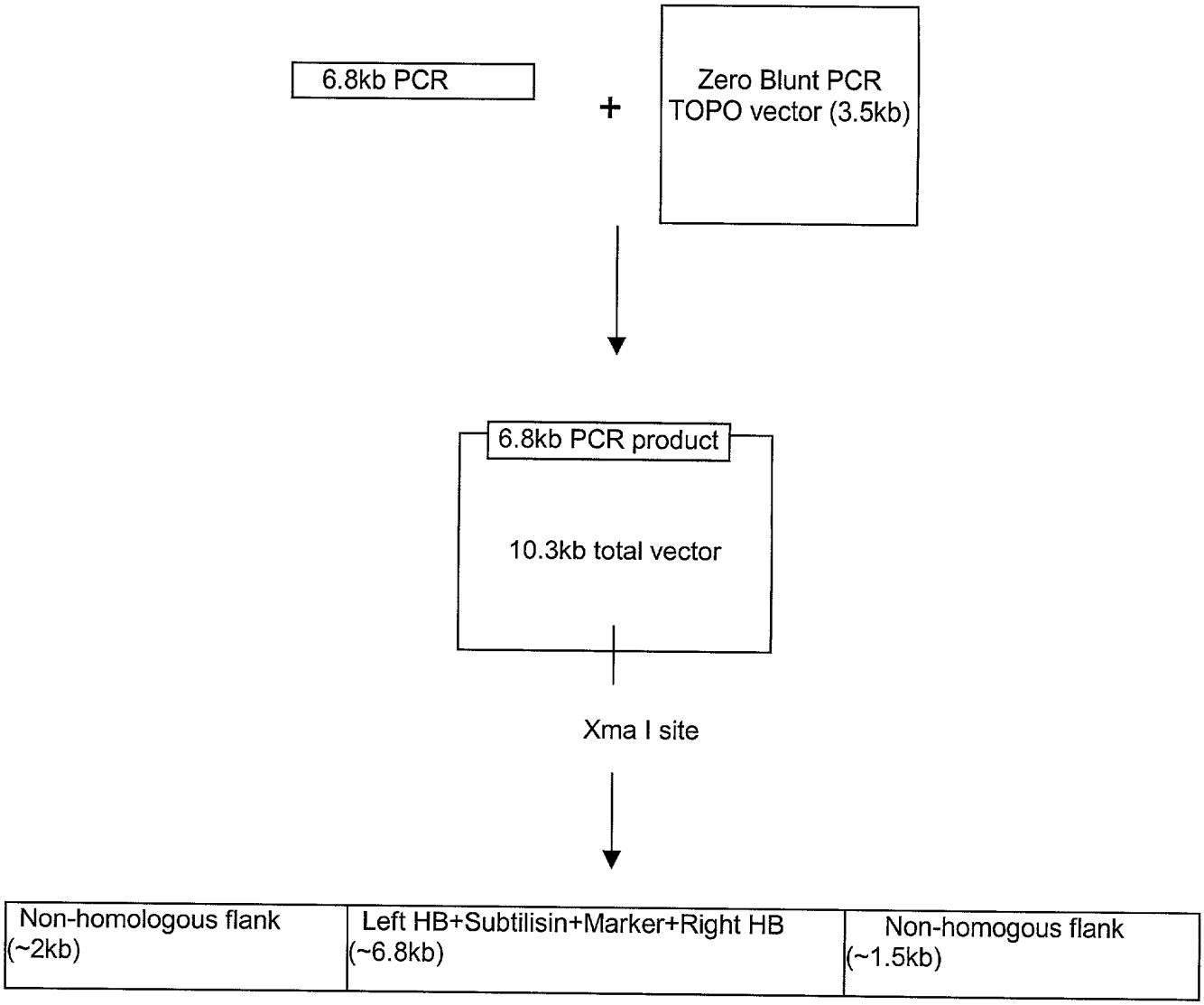

6.8kb PCR
+
Zero Blunt PCR TOPO vector (3.5kb)
6.8kb PCR product
10.3kb total vector
Xma I site
Non-homologous flank (~2kb)
Left HB+Subtilisin+Marker+Right HB (~6.8kb)
Non-homogous flank (~1.5kb)

Figure 12

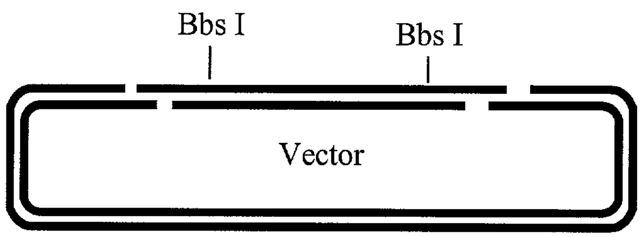


Figure 13

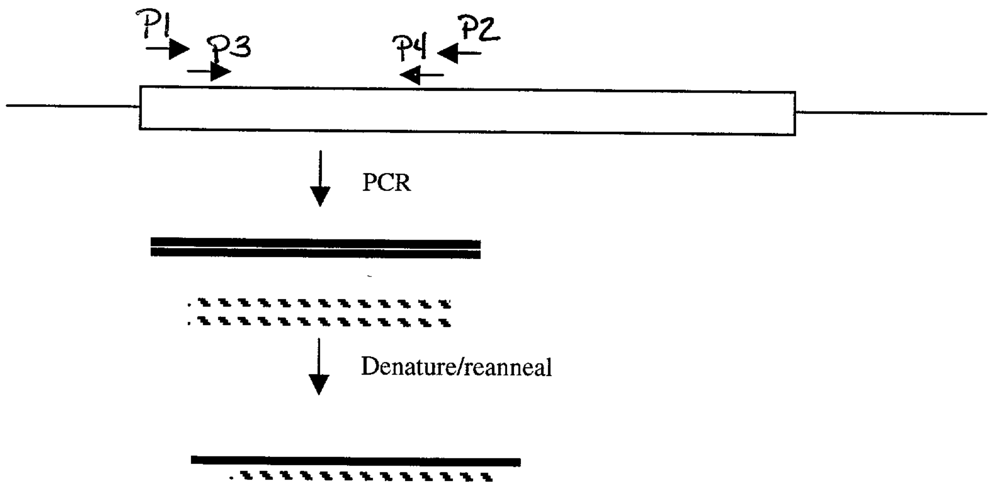
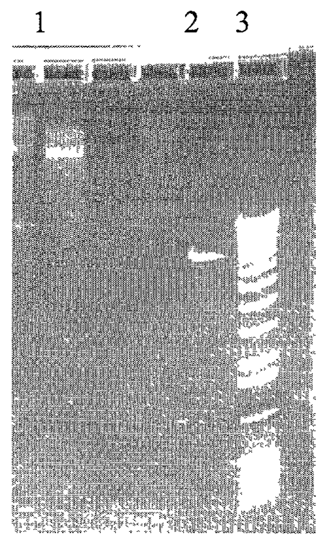


Figure 14



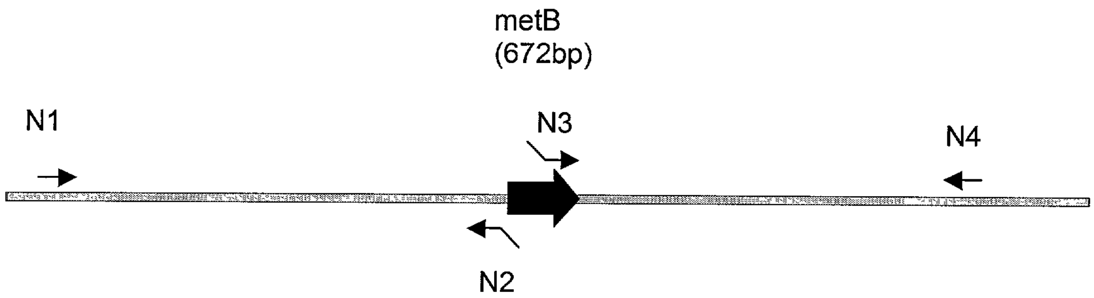


Figure 15